METHOD FOR OBTAINING A. NIGER CULTURES AND THEIR USES FOR PRODUCING FERULIC ACID AND VANILLIC ACID

The present invention relates to the production of ferulic acid and vanillic acid by bioconversion.

Vanillin, which is currently the flavoring most commonly used in the agrofoods industries, can advantageously be produced by bioconversion from ferulic acid or from vanillic acid (which is, itself, a product of bioconversion from ferulic acid), using filamentous fungi.

Thus, European patent application 453 368 in the name of the company PERNOD-RICARD describes the production of natural vanillin by bioconversion from ferulic acid or from vanillic acid in the presence of a filamentous fungus of the Basidiomycete group, *Pycnoporus cinnabarinus*. PCT application WO/96/08576 in the name of INRA describes a two-step bioconversion process which makes it possible to obtain a higher yield. In the first step, ferulic acid is converted into vanillic acid by a filamentous fungus (Ascomycete, Basidiomycete or Actinomycete); the vanillic acid produced is then converted into vanillin by a Basidiomycete.

Ferulic acid, which constitutes the starting product for these bioconversion processes, is one of the major phenol compounds of the plant cell wall. It has been described in monocotyledons, in particular cereals (wheat, maize, etc.), and in dicotyledons of the Chenopodiacea family (beetroot, spinach, etc.). It is generally esterified to the polysaccharides of the plant wall, via arabinose or galactose in beetroot pectins, or arabinose in cereal arabinoxylans.

Ferulic acid is present in diverse agricultural coproducts: for example, wheat bran (residues from

milling), maize bran (residues from semolina production) and beetroot pulps (residues from the sugar industry) contain from 0.6 to 4% of ferulic acid. These products constitute readily available and relatively inexpensive potential sources of ferulic acid.

Although it is theoretically conceivable, in the context of carrying out the bioconversion processes described in application EP 0 453 368 or PCT application WO/96/08576, to produce vanillic acid directly from ferulic acid esterified to the polysaccharides, the bioconversion rate under these conditions is negligible.

The ferulic acid must therefore be released beforehand with ferulate esterases. Depending on the nature of the polysaccharide carrying the ferulic acid, the ferulic acid-saccharide bond is different: ferulic acid forms an ester bond with the O-5 of arabinose in cereal arabinoxylans, whereas, in the case of beetroot pectins, the ferulic acid is carried either by the O-2 of arabinose or, to a lesser extent, by the O-6 of galactose. The release of ferulic acid will therefore involve different enzymes depending on the nature of the bond to be broken: the main ferulate esterases demonstrated in *A. niger* are given in table I below.

Table I

Enzyme	Origin	Preferred substrate
FAEI	*Aspergillus niger*	Arabinose feruloylated at O-2/galactose feruloylated at O-6
FAEII	*Aspergillus niger*	arabinose feruloylated at O-5
FAEIII	*Aspergillus niger*	arabinose feruloylated at O-5
CinnAE	*Aspergillus niger*	arabinose feruloylated at O-2

In addition, the ferulate esterases do not act directly on the plant wall polysaccharides: the bonds which exist between the saccharides in the parietal polysaccharides must be broken beforehand in order for

it to be possible for the ferulic acid to be released by the ferulate esterases. Given the diversity of the parietal polysaccharides, the rupturing of these bonds requires a large number of different enzymatic activities, the main ones of which are: polygalacturonase, rhamnogalacturonase, arabinanase, galactanase, xylanase and glucanase. The hydrolysis products released by these enzymes are, in turn, degraded by osidases (arabinofuranosidase and galactosidase) and ferulic acid is released by ferulate esterases.

Enzymatic mixtures which make it possible to release the ferulic acid present in the parietal polysaccharides of beetroot pulps or of cereal brans are commercially available. However, these enzymatic mixtures do not have sufficient ferulate esterase activity and must be supplemented with ferulate esterases extracted from microbial culture media. In addition, once the ferulic acid has been released, the purification thereof involves many long and expensive steps of liquid/solid and liquid/liquid separation.

However, the inventors have now succeeded in inducing, in *Aspergillus niger*, the production of enzymes with a broad spectrum of activity, allowing not only efficient release of ferulic acid, but also the direct production of natural vanillic acid from agricultural coproducts.

A subject of the present invention is a process for producing *Aspergillus niger* cultures with a broad spectrum of enzymatic activity, characterized in that it comprises culturing at least one *Aspergillus niger* strain in the presence of at least one inducing carbon-containing source chosen from the group consisting of:
- beetroot pulp or at least a soluble fraction thereof rich in feruloylated oligosaccharides, which can be produced by acid hydrolysis;

- a cereal bran, in particular a maize bran, or a mixture of brans of various cereals, or at least a soluble fraction thereof rich in feruloylated oligosaccharides, which can be produced by autoclaving said bran or said mixture.

According to a preferred embodiment of the present invention, said inducing carbon-containing source is present in said culture medium at a concentration of between 1 and 50 grams (dry weight), and preferably between 2.5 and 30 grams, per liter of culture medium.

According to another preferred embodiment of the present invention, the *Aspergillus niger* culture comprises at least the CNCM I-1472 strain, deposited on August 31, 1994, with the CNCM (Collection Nationale de Cultures de Micro-organismes [National Collection of Microorganism Cultures], 26 rue du Docteur Roux, Paris).

A subject of the present invention is also:
- a process for producing an enzymatic preparation with a broad spectrum of activity, characterized in that it comprises culturing at least one *Aspergillus niger* strain according to the process defined above, and recovering the culture supernatant;
- an enzymatic preparation which can be produced using said process.

The culture supernatant can be recovered by any means known per se, such as centrifugation or filtration, which make it possible to separate the *Aspergillus niger* cells from the culture medium. An enzymatic preparation in accordance with the invention may consist of the supernatant itself, or of a concentrate of said supernatant, obtained for example by ultrafiltration or by lyophilization.

A subject of the present invention is also a process for producing free ferulic acid from a feruloylated substrate, which process is characterized in that it comprises bringing said substrate into contact with at least one *Aspergillus niger* culture produced beforehand according to the process in accordance with the invention, or with at least one enzymatic preparation in accordance with the invention, under conditions which allow the release of the ferulic acid by the enzymes present in said culture or said enzymatic preparation.

For the purposes of the present invention, the term "feruloylated substrate" is intended to mean any product containing or consisting of at least one feruloylated polysaccharide and/or at least one feruloylated oligosaccharide. It is in particular any substrate of plant origin comprising feruloylated parietal polysaccharides and/or feruloylated oligosaccharides.

When the plant substrate comprises mainly insoluble parietal polysaccharides, they may advantageously be made more accessible to enzymatic hydrolysis by subjecting them, beforehand, to chemical treatment, such as acid or alkaline hydrolysis, and/or to physical treatment, such as autoclaving or cooking-extrusion.

By way of plant substrates which are particularly advantageous for implementing the present invention, mention will be made in particular:

- of beetroot pulp or the soluble fractions thereof rich in feruloylated oligosaccharides, which can be produced by acid hydrolysis;
- a cereal bran, in particular a maize bran, or a mixture of brans of various cereals, or the soluble fractions thereof rich in feruloylated oligosaccharides, which can be produced by autoclaving.

The feruloylated substrate may be brought into contact with at least one *Aspergillus niger* strain cultured beforehand in accordance with the invention, by adding to the *Aspergillus niger* culture medium an amount of said feruloylated substrate corresponding, for example, to a supply of 0.1 to 50 g of ferulic acid per liter of culture medium. Preferably, the amount of feruloylated substrate added to the culture medium corresponds to a supply of 1 to 20 g, and advantageously to a supply of 5 to 15 g, of ferulic acid per liter of culture medium.

The feruloylated substrate may be brought into contact with at least one enzymatic preparation in accordance with the invention by mixing said enzymatic preparation with said feruloylated substrate, for example in proportions corresponding to a supply, via said substrate, of 0.1 to 40 g of ferulic acid per gram of total proteins of the enzymatic preparation. Preferably, the proportions of the mixture correspond to a supply of 0.2 to 10 g, and advantageously of 0.5 to 5 g, of ferulic acid per gram of total proteins of the enzymatic preparation.

The amount of feruloylated substrate added to the culture medium or to the enzymatic preparation varies in particular depending on the nature of said substrate and of its initial content of esterified ferulic acid. This content can be easily determined using any method known, per se, to those skilled in the art, for example using the method described by SAULNIER et al. [Carbohydrate Research, 272, 241-253, (1995)].

Said feruloylated substrate may be added just once, several times with successive additions, or continuously.

Advantageously, in order to implement the process in accordance with the invention, use will be made of an *Aspergillus niger* culture or an enzymatic preparation

produced in the presence of an inducing carbon-containing source comprising beetroot pulp or at least a fraction thereof rich in feruloylated oligosaccharides, which can be produced by acid hydrolysis, and a feruloylated substrate comprising at least one cereal bran, in particular a maize bran, or at least a fraction thereof rich in feruloylated oligosaccharides, produced by autoclaving.

The ferulic acid produced under these conditions may, if desired, be harvested from the culture medium. However, it is particularly advantageous to carry out the bioconversion of the ferulic acid to vanillic acid directly, with the same *Aspergillus niger* culture, the cells of which have the intracellular enzymes required for this bioconversion.

The ferulic acid or the vanillic acid produced in accordance with the invention may be used per se, for example as antioxidants, or may be used as vanillin precursors in bioconversion processes such as those described in application EP 0 453 368 or PCT application WO/96/08576).

The present invention will be more clearly understood using the further description which follows and which refers to examples of implementation of the process in accordance with the invention.

It should be clearly understood, however, that these examples are given only by way of illustration of the subject of the invention, of which they in no way constitute a limitation.

EXAMPLE 1: ENZYMATIC ACTIVITY OF A. *NIGER* I-1472 CULTURED ON BEETROOT PULP:

A) Production of *Aspergillus niger* I-1472 cultures

The *Aspergillus niger* strain deposited on August 31, 1994, with the Collection Nationale de Cultures de

Micro-organismes [National Collection of Microorganism Cultures], under the number I-1472, was cultured in the presence of agricultural coproducts, or of fractions thereof, as inducing carbon-containing sources for enzymes of interest.

The composition of the culture medium is as follows:

Inducing carbon-containing source	15.00 g/l
Maltose	2.50 g/l
Diammonium tartrate	1.842 g/l
KH_2PO_4	0.20 g/l
$CaCl_2.2H_2O$	0.0132 g/l
$MgSO_4.7H_2O$	0.50 g/l
Yeast extract	0.50 g/l
Tween 80	0.50 g/l

Beetroot pulp is used as the inducing carbon-containing source.

The composition of the beetroot pulp (dry weight) is as follows:

Rhamnose	24 mg/g
Arabinose	209 mg/g
Xylose	17 mg/g
Galactose	51 mg/g
Glucose	211 mg/g
Uronic acids	211 mg/g
Ferulic acid	8 mg/g
Proteins	113 mg/g
Ash	36 mg/g

A control culture is produced on maltose (20 g/L) as the only carbon source. The medium is sterilized by autoclaving for 20 minutes at 120°C. The cultures are grown in 500 ml flasks containing 200 ml of medium. The inoculation is performed with conidiospores (2×10^5

spores/ml). After inoculation, the cultures are incubated at 30°C and subjected to shaking at 120 rpm.

The production of enzymes during the culturing is monitored by assaying the enzymatic activities produced. In order to carry out this assay, an aliquot of the culture medium of approximately 10 ml is removed sterilely each day. This aliquot is then filtered over glass fibers and the enzymatic activities with respect to various substrates are assayed.

B) Enzymatic activity assay

1) Depolymerization enzymes

The enzymatic activities are measured on various pure polysaccharides: galacturonan, carboxymethylcellulose, xylan, type I galactan, arabinan, rhamnogalacturonan.

The reaction medium contains 0.9 ml of substrate solution at 1 g/l in 50 mmol/L acetate buffer, pH 4.5, and 0.1 ml of culture filtrate. The mixture is incubated for 10 minutes at 40°C. The reducing ends released are assayed in microplates with copper sulfate [NELSON, J. Biol. Chem. 153, 375-380 (1944); STURGEON, Methods in Plant Biochemistry, 2, 1-37 (1990)]. The constituent saccharide of the polysaccharide is used as a standard: galacturonic acid for the assays on galacturonan and rhamnogalacturonan, glucose for the assays of carboxymethylcellulose, xylose for the assays on xylan, galactose for the assays on galactan and arabinose for the assays on arabinan.

2) Osidase activities

The osidase activities are measured on *para*-nitrophenyl-β-D-galactopyranoside and *para*-nitrophenyl-α-L-arabinofuranoside (SIGMA).

The reaction medium contains 0.1 ml of substrate solution at 4 mmol/L in 50 mmol/L acetate buffer, pH 4.5, and 0.1 ml of culture supernatant filtrate. The

mixture is incubated for 20 minutes at 40°C. 0.6 ml of sodium carbonate are then added in order to inhibit the enzymatic activity and allow the colorimetric reaction to develop. The concentration of *para*-nitrophenol released is calculated from the optical density of the mixture read at λ = 400 nm.

3) Ferulate esterase activities

The ferulate esterase activities are measured on various feruloylated oligomers: 5-O-(transferuloyl)-L-Araf (determination of FA activity) and O-β-D-Xylp(1→2)-[5-O-transferuloyl)-α-L-Araf] (determination of XFA activity) isolated from maize bran [SAULNIER et al., Carbohydrate Research, 272, 241-253, (1995)] and [2-O-(transferuloyl)α-L-Araf (determination of FA_2 activity) isolated from beetroot pulp [KROON et al., Carbohydrate Research, 300, 351-354, (1997)]. The assay is carried out at the optimum for the specific activities of the other enzymes.

The reaction medium contains 100 µl of substrate solution at 70 nmol/L, 80 µl of 0.1 mol/L MOPS (3-[N-morpholino]propanesulfonic acid] buffer, pH 6, and 20 µl of culture filtrate. The mixture is incubated for 1 hour at 40°C. At times: 0, 15, 30, 45, 60 minutes, 10 µl of reaction medium are removed and poured into MOPS buffer. The optical densities are read at 286 and 323 nm.

The reaction medium contains both esterified ferulic acid and ferulic acid released by the enzyme. Their respective amounts are calculated from the optical densities using the molar extinction coefficients determined beforehand at pH 6: ε_{286} = 14176 $L.mol^{-1}.cm^{-1}$, ε_{323} = 10350 $L.mol^{-1}.cm^{-1}$ for the free ferulic acid, and ε'_{286} = 12465 $L.mol^{-1}.cm^{-1}$, ε'_{323} = 19345 $L.mol^{-1}.cm^{-1}$ for the esterified ferulic acid.

All the enzymatic activities are expressed as nkat/ml, which corresponds to the amount of enzyme required to release one nmol of product per second and per ml, under the pH and temperature conditions defined above.

C) Results

The production of enzymes which degrade the parietal polysaccharides was monitored during the culturing of *A. niger* on beetroot pulp and during the control culturing on maltose. The enzymatic activities were measured as described above.

1) Depolymerization enzymes and osidases

The results obtained for the depolymerization enzymes and the osidases are illustrated by figure 1, which represents the hydrolytic activity for each substrate tested, as a function of the number of days of culturing.

Legend to figure 1:
Figure 1A: Induction on beetroot pulp;
Figure 1B: Induction on maltose;
● : Galactan;
■ : Arabinan;
▲ : Polygalacturonic acid;
○ : Carboxymethylcellulose;
▫ : Xylan;
▼ : Rhamnogalacturonan;
× : pNPGalactoside;
· : pNPArabinoside.

When maltose only is used as the carbon-containing source in the *A. niger* I-1472 cultures, very few enzymatic activities which are active on the parietal polysaccharides are released. On the other hand, the presence of beetroot pulp as the inducing carbon-containing source leads to much greater synthesis of enzymes which degrade parietal polysaccharides. A broad spectrum of enzymes is present in the culture

supernatant. In addition, the synthesis of these enzymes is rapid and it is observed that the enzymatic activities reach their maximum value from the 3rd day of culturing. For this reason, ferulate esterase activities were sought in the culture supernatants at 3 days.

2) Ferulate esterases

The ferulate esterase activities in the culture supernatants are given in the following table II:

Table II

Inducing carbon-containing source	Activity (nkat/ml)		
	FA	FAX	FA_2
Maltose control	0	0	0
Beetroot pulp	1.1	0.5	0

Low FA and FAX activity is therefore induced when *A. niger* I-1472 is cultured on beetroot pulp.

EXAMPLE 2: ENZYMATIC ACTIVITIES OF *A. niger* I-1472 CULTURED ON BEETROOT PULP HYDROLYSATES

The beetroot pulp undergoes two successive hydrolyses with trifluoroacetic acid in order to extract the feruloylated oligomers therefrom, according to the following protocol [RALET et al. Carbohydrate Research, 263, 227-241 (1994)]:

800 g of beetroot pulp are mixed, in a proportion of 20 g/L, with a 50 mmol/L solution of trifluoroacetic acid. The mixture is maintained at 100°C for 1 h 30. The soluble fraction is collected and precipitated by adding 4 volumes of ethanol. The ethanol-soluble fraction is collected and constitutes hydrolysate 1.

The precipitate is treated with trifluoroacetic acid (150 mmol/L) for 6 hours at 100°C. The soluble fraction is collected and precipitated by adding 4 volumes of

ethanol. The ethanol-soluble fraction is collected and constitutes hydrolysate 2.

The fractions termed: "hydrolysate 1" and: "hydrolysate 2" are obtained with yields of 280 mg/g for hydrolysate 1 and of 76 mg/g for hydrolysate 2, and have the following compositions (dry weight):

Hydrolysate:	1	2
Rhamnose	6 mg/g	54 mg/g
Arabinose	535 mg/g	129 mg/g
Xylose	2 mg/g	2 mg/g
Galactose	16 mg/g	187 mg/g
Glucose	85 mg/g	42 mg/g
Uronic acids	17 mg/g	188 mg/g
Ferulic acid	10 mg/g	12 mg/g
Proteins	12 mg/g	8 mg/g
Ash	64 mg/g	86 mg/g

Hydrolysate 1 or 2 is then used as the inducing carbon-containing source in an *A. niger* I-1472 culture and the enzymes produced by the fungus are measured according to the protocols described in example 1.

1) Depolymerization enzymes and osidases

The results obtained for the depolymerization enzymes and the osidases are illustrated by figure 2, which represents the hydrolytic activity for each substrate tested, as a function of the number of days of culturing.

Legend to figure 2:
Figure 2A: Induction on hydrolysate 1;
Figure 2B: Induction on hydrolysate 2;
● : Galactan;
■ : Arabinan;
▲ : Polygalacturonic acid;
○ : Carboxymethylcellulose;
ª : Xylan;
▼ : Rhamnogalacturonan;

× : pNPGalactoside;

· : pNPArabinoside.

When beetroot pulp hydrolysate 1 or 2 is used as the inducing carbon-containing source in the *A. niger* I-1472 cultures, the production of enzymes which degrade the parietal polysaccharides is lower than in the cultures on beetroot pulp. The hydrolysates therefore induce the synthesis of lower amounts of enzymes. In addition, it is observed that the enzymatic activities reach their maximum value after 4 days of culturing on hydrolysate 1 and after 3 days on hydrolysate 2.

2) Ferulate esterases

The ferulate esterase activities were measured in the supernatants from the 3rd day of culturing for hydrolysate 2, and from the 4th day of culturing for hydrolysate 1. The results are illustrated by table III below.

Table III

Inducing carbon-containing source	Activity (nkat/ml)		
	FA	FAX	FA_2
Maltose control	0	0	0
Hydrolysate 1	0.6	0	0.2
Hydrolysate 2	0	0	0

Low FA and FA_2 activities are induced when *A. niger* I-1472 is cultured on hydrolysate 1 from beetroot pulp, which is rich in ferulic acid esterified to arabinose.

EXAMPLE 3: ENZYMATIC ACTIVITIES PRODUCED BY *A. niger* I-1472 CULTURED ON MAIZE BRAN

A. niger I-1472 is cultured in the presence of maize bran as the inducing carbon-containing source, and the enzymes produced by the fungus are measured according to the protocols described in example 1.

The composition of the maize bran (dry weight) is as follows:

Rhamnose	0 mg/g
Arabinose	154 mg/g
Xylose	276 mg/g
Galactose	51 mg/g
Glucose	248 mg/g
Uronic acids	42 mg/g
Ferulic acid	31 mg/g
Proteins	50 mg/g

1) Depolymerization enzymes and osidases

The results obtained for the depolymerization enzymes and the osidases are illustrated by figure 3, which represents the hydrolytic activity for each substrate tested, as a function of the number of days of culturing.

Legend to figure 3:

- ● : Galactan;
- ■ : Arabinan;
- ▲ : Polygalacturonic acid;
- ○ : Carboxymethylcellulose;
- ª : Xylan;
- ▼ : Rhamnogalacturonan;
- × : pNPGalactoside;
- · : pNPArabinoside.

These results show that, in the case of the depolymerization enzymes and of the osidases, the enzymatic activities induced during culturing in the presence of maize bran are lower than those induced in the presence of beetroot pulp.

2) Ferulate esterases

The ferulate esterase activities measured in the supernatant after 3 days of culturing are, respectively, 4.6 nkat/ml for FA and 4.9 nkat/ml for

FAX. A clear induction of the FA and FAX activities by the maize bran is observed.

EXAMPLE 4: ENZYMATIC ACTIVITIES PRODUCED BY *A. niger* I-1472 CULTURED ON THE AUTOCLAVED MATERIAL DERIVED FROM MAIZE BRAN

The maize bran undergoes treatment by autoclaving in order to extract the feruloylated oligomers therefrom, according to the following protocol:

The bran is suspended in water (in a proportion of 100 g of bran per liter) and then autoclaved at 160°C for 60 minutes. The autoclaved material is centrifuged for 10 minutes at 20 000 rpm and then filtered over G3 sintered glass (pore size 15-40 µm; SCHOTT). The filtered supernatant is lyophilized. The final product, named hereinafter "autoclaved material from maize bran", is obtained with a yield of 600 mg/g and has the following composition (dry weight):

Rhamnose	0 mg/g
Arabinose	208 mg/g
Xylose	386 mg/g
Galactose	73 mg/g
Glucose	39 mg/g
Uronic acids	47 mg/g
Ferulic acid	34 mg/g
Proteins	8 mg/g
Ash	8 mg/g

The autoclaved material is then used as the inducing carbon-containing source for the *A. niger* I-1472 culture and the enzymes are measured in the culture supernatant according to the protocols described in example 1.

1) Depolymerization enzymes and osidases

The results obtained for the depolymerization enzymes and the osidases are illustrated by figure 4, which represents the hydrolytic activity for each substrate tested, as a function of the number of days of culturing.

Legend to figure 4:

- ● : Galactan;
- ■ : Arabinan;
- ▲ : Polygalacturonic acid;
- ○ : Carboxymethylcellulose;
- ª : Xylan;
- ▼ : Rhamnogalacturonan;
- × : pNPGalactoside;
- · : pNPArabinoside.

It is noted, in particular, that the xylanase activity induced in the presence of maize bran hydrolysate is much higher than that induced when native maize bran is used as the inducing carbon-containing source.

2) Ferulate esterases

The ferulate esterase activities measured in the supernatant after 5 days of culturing are, respectively, 9.7 nkat/ml for FA and 10.6 nkat/ml for FAX; an induction which is even more significant than that observed in the case of the cultures produced in the presence of maize bran is therefore observed.

EXAMPLE 5: RELEASE OF FERULIC ACID BY THE *A. niger* I-1472 ENZYMES

The culture supernatant from *A. niger* I-1472 cultured in the presence of beetroot pulp as described in example 1 above, and concentrated 20 times by lyophilization, was used as the source of enzymes for releasing the ferulic acid present either in beetroot pulp or in the autoclaved material from maize bran.

This release was compared with the release of ferulic acid by commercially available enzymes: SP 584 (NOVO) in the case of the beetroot pulp and NOVOZYME 342 (NOVO) in the case of the autoclaved material from maize bran.

Table IV below illustrates the comparison between the enzymatic activities present in the 4 enzyme preparations used:

Table IV

	Specific activity (nkat/mg)			
Substrate	Supernatant from *A. niger* I-1472 on beetroot pulp	Supernatant from *A. niger* I-1472 on autoclaved material from maize bran	SP 584	NOVOZYM 342
Arabinan	120.6	1.9	291.1	7.2
Xylan	125.5	93.9	62.2	104.3
Galactan	32.9	1.8	943.9	2.8
Rhamnogalacturonan	53.4	4.9	256.7	Nd
CMC	13.6	12.2	4.7	24.9
Polygalacturonic acid	86.0	1.5	2400.2	0.4
pNP-Rha	12.9	nd	0.2	0.0
pNP-Gal	19.8	3.8	84.8	0.0
pNP-Ara	266.6	27.5	619.5	0.3
XFA	9.2	85.9	0.1	0.8
FA	5.8	90.4	0.1	0.2
FA2	2.4	nd	0.3	Nd

nd: not determined

A) Release of the ferulic acid contained in beetroot pulp

The enzymatic degradation of beetroot pulp was carried out in the presence of 10 mg of proteins (SP 584 or *A. niger* I-1472 enzymes) per g of dry pulp, i.e. 10 mg of proteins per 8 mg of esterified ferulic acid initially present in the beetroot pulp. After hydrolysis for 24 h, the amount of ferulic acid released by SP 584 represents 50% of this initial amount of ferulic acid, and the amount of ferulic acid released by the *A. niger* I-1472 enzymes represents 40% of this initial amount. The enzymes secreted by

A. niger I-1472 are therefore slightly less efficient than SP 584 in releasing the ferulic acid present in beetroot pulp.

B) Release of the ferulic acid contained in the autoclaved material from maize bran

The enzymatic degradation of the autoclaved material from maize bran was carried out in the presence of 10 mg of proteins (NOVOZYM 342 or *A. niger* I-1472 enzymes) per g of dry autoclaved material, i.e. 10 mg of proteins per 34 mg of esterified ferulic acid initially present in the autoclaved material from maize bran. After hydrolysis for 24 h, the amount of ferulic acid released by NOVOZYM 342 represents 33% of this initial amount of ferulic acid, and the amount of ferulic acid released by the *A. niger* I-1472 enzymes represents 95% of this initial amount. The enzymes secreted by *A. niger* I-1472 are therefore much more efficient than NOVOZYM 342 in releasing the ferulic acid contained in the autoclaved material from maize bran.

EXAMPLE 6: DIRECT BIOCONVERSION OF THE FERULIC ACID PRESENT IN AGRICULTURAL COPRODUCTS OR FRACTIONS THEREOF, TO VANILLIC ACID, BY *A. niger* I-1472 CULTURED IN THE PRESENCE OF MAIZE BRAN

A. niger I-1472 was cultured in the presence of maize bran as the carbon-containing source inducing enzymes capable of degrading the parietal polysaccharides. The production of vanillic acid was monitored using, respectively, the autoclaved material from maize, or beetroot pulp or hydrolysates thereof as the plant substrate acting as the source of ferulic acid.

Conditions for bioconversion of ferulic acid to vanillic acid

The *A. niger* I-1472 strain was cultured under the same culture conditions as those described previously for the production of enzymes. The cultures are produced in laboratory bioreactors (2 liter volume) with mechanical shaking.

At the optimum point of production of the enzymes (generally on the 3rd day of incubation of the culture), the plant substrate acting as the source of esterified ferulic acid is added to the *A. niger* I-1472 culture in an amount corresponding to 0.3 to 1.5 g of ferulic acid per liter of culture and per day.

The bioconversion of the released ferulic acid to vanillic acid is monitored by HPLC. The HPLC analysis is performed on aliquots of the culture medium removed at regular time points and filtered over glass fibers.

The analytical conditions are as follows: MERCK LICHROSPHER 100 RP18 HPLC column (15 µm, 125 × 4 mm), maintained at 30°C; flow rate of 0.75 ml/minute; UV detection at 280 nm.

Elution solvent: A: 0.01% acetic acid in water; B: methanol. The elution profile is as follows: 20% of solvent B for 4 minutes; linear gradient of 20 to 40% of solvent B for 24 minutes; 100% of solvent B for 2 minutes; return to 20% of solvent B and equilibration of the column for 5 minutes.

The results obtained at the optimum point of production of vanillic acid from the various sources of ferulic acid used are given in table V below. The amount of ferulic acid consumed corresponds to the difference between the total amount of ferulic acid added to the culture in the form of plant substrate, and the total

amount of ferulic acid present in the culture at the time of assay (it is not possible to measure, directly, the amount of ferulic acid released since, in the presence of the *A. niger* cells, this ferulic acid is immediately converted to vanillic acid as it is released).

Table V

Source of ferulic acid	Total amount of bound ferulic acid added to the culture (mg/l)	Amount of ferulic acid consumed (mg/l)	Vanillic acid produced (mg/l)
Native maize bran	600	0	0
Autoclaved material from maize bran	4240	3460	1400 (after 7 days of culture)
Beetroot pulp	600	0	0
Beetroot pulp hydrolysate 1	1680	1160	350 (after 6 days of culture)
Beetroot pulp hydrolysate 2	4240	3730	950 (after 7 days of culture)

Maximum production of vanillic acid is observed when the plant substrates rich in feruloylated oligosaccharides (beetroot pulp hydrolysates and autoclaved material from maize bran) are used as sources of ferulic acid. The ferulic acid is therefore released by the induced *A. niger* I-1472 enzymes, and immediately biotransformed into vanillic acid by the intracellular enzymes, in a proportion of 1400 mg/l in 7 days with a molar yield of 46% relative to the ferulic acid consumed, and of 950 mg/L in 7 days with a molar yield of 29% relative to the ferulic acid consumed, when the autoclaved material from maize bran and hydrolysate 2 (rich in ferulic acid esterified to galactose), respectively, are used as the source of ferulic acid.

EXAMPLE 7: DIRECT BIOCONVERSION OF THE FERULIC ACID PRESENT IN AGRICULTURAL COPRODUCTS OR FRACTIONS THEREOF, TO VANILLIC ACID, BY *A. NIGER* I-1472 CULTURED IN THE PRESENCE OF BEETROOT PULP

In this example, the enzyme-inducing carbon-containing source is beetroot pulp. Maize bran, the autoclaved material from maize, beetroot pulp or hydrolysate 1 or 2 thereof is used as the source of ferulic acid.

The production of ferulic acid and of vanillic acid by *A. niger* I-1472 is monitored as indicated in Example 6 above.

The results obtained at the optimum point of production of vanillic acid from the various sources of ferulic acid used are given in table VI below.

Table VI

Source of ferulic acid	Total amount of bound ferulic acid added to the culture (mg/l)	Total amount of ferulic acid consumed (mg/l)	Vanillic acid produced (mg/l)
Native maize bran	600	0	0
Autoclaved material from maize bran	3750	3290	2200 (after 7 days of culture)
Beetroot pulp	600	350	50 (after 6 days of culture)
Beetroot pulp hydrolysate 1	900	650	150 (after 4 days of culture)
Beetroot pulp hydrolysate 2	900	770	270 (after 4 days of culture)

These results show that the use of beetroot pulp as the inducing carbon-containing source produces results for direct bioconversion which are better than those observed when maize bran is used as the inducing carbon-containing source; this is in agreement with the results regarding the potentialities of the *A. niger* I-1472 enzymes induced under these conditions (examples 1 and 2). Specifically, in the case of the cultures produced in the presence of beetroot pulp, a spectrum

of enzymatic activities which is broader, and activity levels which are higher, than in the case of the cultures produced in the presence of maize bran are observed for the depolymerization enzymes and the osidases; this allows the release of a greater amount and of a greater variety of feruloylated oligosaccharides used as substrate by the ferulate esterases.

In addition, maximum production of vanillic acid is obtained when the autoclaved material from maize bran is used as the plant substrate acting as the source of ferulic acid. In the cultures produced in the presence of beetroot pulp as the carbon-containing source inducing the depolymerization enzymes and the osidases, the addition of autoclaved material from maize also induces the ferulate esterase activities.

The ferulic acid released by the induced *A. niger* I-1472 enzymes is then very efficiently biotransformed into vanillic acid by the intracellular enzymes, in the proportion of 2 200 mg/L in 7 days with a molar yield of 77% relative to the ferulic acid consumed.

It should be noted that the bound ferulic acid present in the beetroot pulps, and hydrolysates thereof, used as the plant substrate acting as the source of ferulic acid was also directly bioconverted to vanillic acid, although at a lower level.

EXAMPLE 8: DIRECT BIOCONVERSION OF THE FERULIC ACID PRESENT IN THE AUTOCLAVED MATERIAL FROM MAIZE, TO VANILLIC ACID, BY *A. NIGER* I-1472 CULTURED IN THE PRESENCE OF BEETROOT PULP HYDROLYSATES

In this example, the enzyme-inducing carbon-containing sources are beetroot pulp hydrolysates 1 and 2. The autoclaved material from maize is used as the plant substrate acting as the source of ferulic acid.

The production of ferulic acid and of vanillic acid by *A. niger* I-1472 is monitored as indicated in example 6 above.

The results obtained at the optimum point of production of vanillic acid are illustrated by table VII below:

Table VII

Inducing carbon-containing source	Total amount of bound ferulic acid added to the culture (mg/l)	Total amount of ferulic acid consumed (mg/l)	Vanillic acid produced (mg/l)
Hydrolysate 1 (rich in ferulic acid esterified to arabinose)	3300	3150	1260 (after 7 days of culture)
Hydrolysate 2 (rich in ferulic acid esterified to galactose)	3300	3180	1550 (after 7 days of culture)

The production of vanillic acid by *A. niger* I-1472, from the ferulic acid contained in the autoclaved material from maize, is 1260 mg/L after 7 days (i.e. a molar yield of 46% relative to the ferulic acid consumed) when hydrolysate 1 is used as the inducing carbon-containing source, and 1550 mg/L after 7 days (i.e. a molar yield of 56% relative to the ferulic acid consumed) when hydrolysate 2 is used as the inducing carbon-containing source.